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# Non-photopic and photopic visual cycles enable different phases of cone photoreceptor-mediated vision

**Authors:** Rebecca Ward<sup>1</sup>, Joanna J. Kaylor<sup>2</sup>, Diego F. Cobice<sup>3</sup>, Dionissia A. Pepe<sup>4</sup>, Eoghan M. McGarrigle<sup>4</sup>, Susan E. Brockerhoff<sup>5,6</sup>, James B. Hurley<sup>5,6</sup>, Gabriel H. Travis<sup>2,7</sup> & Breandán N. Kennedy<sup>1</sup>

## Affiliations:

1. UCD School of Biomolecular & Biomedical Science, UCD Conway Institute, University College Dublin, D04 V1W8, Ireland
2. Jules Stein Eye Institute, University of California, Los Angeles School of Medicine, Los Angeles, CA 90095, USA
3. Mass Spectrometry Centre, School of Biomedical Sciences, Biomedical Sciences Research Institute, Ulster University, Coleraine, BT52 1SA, Northern Ireland
4. Centre for Synthesis and Chemical Biology, UCD School of Chemistry, University College Dublin, D04 V1W8, Ireland.
5. Department of Biochemistry, University of Washington, Seattle, WA 98109, USA.
6. Department of Ophthalmology, University of Washington, Seattle, WA 98109, USA
7. Department of Biological Chemistry, University of California, Los Angeles School of Medicine, Los Angeles, CA 90095, USA

**Running Title:** Chemical Biology of Visual Cycles Enabling Cone Vision

**Correspondence:** Breandán Kennedy, UCD School of Biomolecular & Biomedical Science, UCD Conway Institute, University College Dublin, Belfield, Dublin D04 V1W8, Ireland.  
[brendan.kennedy@ucd.ie](mailto:brendan.kennedy@ucd.ie)

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## Abstract

Cone photoreceptors in the retina enable vision over a wide range of light intensities. However, the processes enabling cone photopic vision are not adequately understood. Photons isomerize the 11-*cis*-retinaldehyde (11cRAL) chromophore, complexed with opsins, to all-*trans*-retinaldehyde (atRAL) which dissociates. Restoration of light sensitivity occurs when unliganded opsin recombines with 11cRAL. Conversion of free atRAL into 11cRAL involves co-operation with other retinal cells. Chromophore regeneration of cone photopigments may require the retinal pigment epithelium (RPE) and/or retinal Müller glia. In the RPE, isomerization of all-*trans*-retinyl esters (atRE) to 11-*cis*-retinol (11cROL) is mediated by Rpe65. Two additional retinoid isomerases are reported in Müller cells, dihydroceramide desaturase-1 (DES1) and retinal

G-protein-coupled receptor (RGR) opsin. The retinol-isomerase activities of Rpe65 and Des1 are inhibited by emixustat and fenretinide, respectively. Here, the effects of these visual cycle inhibitors on immediate, early and late phases of cone photopic vision was assessed. In zebrafish larvae raised under cyclic light conditions, late cone photopic vision was significantly impaired by fenretinide, whereas emixustat-treated zebrafish had unexpectedly normal vision. In contrast, emixustat-treated larvae raised under extensive dark-adaption display significantly attenuated immediate photopic vision concomitant with significantly reduced 11cRAL. Following 30 minutes of light, early photopic vision recovered, despite 11cRAL levels remaining significantly reduced. Defects in immediate cone photopic vision was rescued in emixustat- or fenretinide-treated larvae following 9-*cis*-retinaldehyde (9cRAL) supplementation.

Zebrafish *des1* or *rlbp1b* were not required for photopic vision. In summary, we elucidate differential requirements of the non-photopic and photopic visual cycles for distinct phases of cone photopic vision.

### **Introduction**

Photopic vision is the physiological function mediating vision in well-lit conditions. Daytime vision requires cone photoreceptors to remain functional in the presence of light. This capability requires regeneration of cone photo-pigments in the retina prior to and during light onset. Our understanding of the biological process contributing to immediate, early and late phases of cone photopic vision is inadequate.

### ***Visual Cycles in RPE and Müller cells***

Sustained vision in vertebrates depends upon a supply of 11-*cis*-retinaldehyde (11cRAL) chromophore to regenerate photobleached visual pigments. The canonical visual cycle is the enzymatic pathway in the retinal pigment epithelium (RPE) that recycles visual chromophore (1,2). The retinoid isomerase in RPE cells is Rpe65, which converts all-*trans* retinyl esters (atREs), synthesized by lecithin-retinol acyltransferase (LRAT), to 11-*cis*-retinol (11cROL) (3-5). Null mutations in the mouse *Rpe65* or *Lrat* gene cause the virtual absence of retinoids within the neural retina (3,6). *Rpe65*<sup>-/-</sup> mice exhibit massive accumulation of atREs in the RPE, while retinoids are almost undetectable in RPE of *Lrat*<sup>-/-</sup> mice (7). *Rpe65* mutations in humans cause the inherited blinding disease, Leber congenital amaurosis (8). The last step in the RPE visual cycle is oxidation of 11cROL to 11cRAL, which is released into the extracellular space for uptake by photoreceptors. Notably, there is still uncertainty as to if, and when, the RPE65 visual cycle contributes to cone photopic vision.

Furthermore, under daylight conditions, the estimated rate of 11cRAL synthesis by RPE cells is much slower than the rate of chromophore consumption by rods and cones (9). The rod photoresponse saturates in bright light, leaving vision mediated only by cones. Despite the non-contribution of rods to daylight vision, rhodopsin continues to photoisomerize in bright-light exposed retinas. Cones must therefore compete with rods for chromophore when demand is highest. As a possible adaptation, cones can regenerate cone-opsin pigments by uptake of

either 11cRAL or 11cROL, while rods can only regenerate rhodopsin by uptake of 11cRAL (5,10,11). Müller glial cells of the neural retina are the likely source of this 11cROL (12). Dihydroceramide desaturase (Des1) and multifunctional *O*-acyltransferase (MFAT) represent another retinol isomerase—retinyl-ester synthase pair, present in Müller cells, which may mediate 11cROL synthesis. The ‘*isomerase*’ activity of Des1—MFAT is present in retinas from cone-dominant chickens and ground squirrels, but undetectable in retinas from rod-dominant mice and cattle (9). This activity correlated with the presence of 11-*cis*-retinyl esters (11cREs) in the neural retina, which are undetectable in mice and cattle retinas. Interestingly, synthesis of 11cROL by DES1 is increased through interactions with cellular retinaldehyde binding protein (CRALBP; encoded by *RLBP1*) an 11cRAL/11cROL carrier protein expressed in the RPE and Müller glia (6). In contrast to the evidence supporting a role for DES1 in cone chromophore regeneration, a recent study has shown that recovery of cone electrophysiological sensitivity in isolated retinas following a photobleach was similar in *Des1*<sup>+/+</sup> and Müller-cell conditional *Des1*<sup>-/-</sup> mutant mice (13), suggesting Müller Des1 plays no role in the recovery of mouse cone electroretinograms. However, uncertainty remains regarding the overall contribution of DES1 to the cone visual cycle as significant DES1 activity remained in retinal lysates of these Müller-cell *Des1*<sup>-/-</sup> mice. There is also conjecture regarding the physiological role of 9-*cis*-retinaldehyde (9cRAL) in photopic vision as cone-dominant animals have higher levels of 9-*cis*-retinoids and DES-1 generates higher levels of 9cRAL than 11cRAL (6,14). Additionally, the cone electrophysiological studies were conducted in rod-dominant mice whereas DES1 was identified in Müller cells from cone-dominant chicken. Thus, uncertainty remains as to whether species-specific retinoid isomerase pathways exist to meet cone chromophore demands.

A third mechanism postulated to replenish cones with chromophore is the photopic visual cycle, a process that regenerates 11cRAL by a light-dependent mechanism. The non-visual opsin, retinal G protein-coupled receptor (RGR) opsin is present in RPE and Müller cells. Previous reports show RGR-opsin is integral to light-dependent biochemical formation of 11cRAL that reconstitutes rhodopsin visual pigment in rods (15). In cones, on exposure to visible light, retinol

dehydrogenase-10 (RDH10) assists RGR-opsin in a light-dependent conversion of atROL to 11cROL (12). Indeed, RGR-opsin appears essential for the recovery of cone electrophysiological sensitivity as retinas from wild-type mice under sustained background illumination maintained cone sensitivity, while *Rgr*<sup>-/-</sup> mice exhibited diminishing cone sensitivity during light exposure (12). Notably, these studies of DES1 and RGR-opsin assess cone electroretinography (ERG) and not cone vision. It is well recognized that electrophysiology does not absolutely correlate to functional vision. For example, patients receiving gene therapy for RPE65 defects show no improvement in ERG but show significant improved ability to navigate an obstacle course (16). Thus, the contribution of DES1 and RGR-opsin to cone photopic vision is unknown.

### ***In Vivo Cone Models***

Most visual cycle knowledge was ascertained by studying retinæ of rod dominant mice. Consequently, investigations of the visual cycles enabling cone vision are less advanced. Zebrafish larvae provide novel opportunities to study cone photopic vision and the supporting visual cycle processes (17) due to easy genetic and pharmacological manipulation, and cone-dominant vision until 15 dpf, the stage when rods become functional (18,19). Previous studies investigating visual cycle components in zebrafish relied on transient morpholino knockdown. Zebrafish *rpe65a* knockdown significantly reduced, but did not completely attenuate 11cRAL synthesis, supporting the presence of an alternate pathway for chromophore synthesis in zebrafish retinæ (17). Knockdown of either *Cralbpa* or *Cralbp* in zebrafish resulted in reduced visual behavior (20) and reduced light sensitivity (21).

### ***Visual Cycle Modulation***

Pharmacological modulators that inhibit or complement the visual cycles are potential treatments for retinal disease (22,23). Following photoisomerization, released atRAL must be delivered to RPE/Müller cells to regenerate 11cRAL to restore light sensitivity. Impaired atRAL clearance from photoreceptors leads to accumulation of bis-retinoid *N*-retinylidene-*N*-retinylethanolamine (A2E), a toxic by-product of atRAL and a pathological hallmark of AMD and Stargardt disease (24). Emixustat (ACU-4429) competitively inhibits Rpe65 and hinders

retinaldehyde-mediated destruction of photoreceptors by acting as a retinaldehyde scavenger (23). Inhibition of chromophore synthesis gives rise to unliganded ‘noisy’ opsins that stimulate the transduction pathway in the absence of light, greatly decreasing photoreceptor sensitivity (25). In *Rpe65*<sup>-/-</sup> mice, chronic activation of signal transduction may cause photoreceptor degeneration (26). Free opsin can be targeted by chromophore-replacement therapy with 9cRAL, which bypasses the visual cycle defect to form isorhodopsin or iso-cone-opsins. Following 9cRAL treatment, light sensitivity is restored long-term in *Rpe65*<sup>-/-</sup> mice with attenuated accumulation of atREs. Fenretinide (4-HPR), a derivative of retinoic acid is reported to inhibit Des1 without affecting Rpe65 activity. Fenretinide also competes with retinol for binding to retinol-binding protein (RBP4) in blood (27-29), causing a mild vitamin A deficiency. A1120 is a non-retinoid RBP4 antagonist which in mice reduces serum retinol levels in a dose-dependent manner (30) and reduces lipofuscin accumulation in ABCA4<sup>-/-</sup> mice through reduction of serum RBP4 and visual retinoids (31). Unlike fenretinide, A1120 does not act as a retinoic acid receptor alpha agonist (30). A1120 does not affect RPE65 isomerohydrolase activity in *ex vivo* experiments (31).

Previous visual cycle inhibition studies focused on measuring retinal function (*e.g.* electrophysiology) as the primary endpoint. Reduced cone photosensitivity was observed in live mice and isolated retinas treated with visual cycle inhibitors (28). To our knowledge, no previous studies investigated the relationship between visual cycle modulation and cone photopic vision. In the current study, we used visual behavior assays, visual cycle inhibitors and genetic approaches in zebrafish to understand the mechanisms regulating immediate (at light onset following dark adaptation, 0 minutes), early (after 30 minutes light adaptation) or late (after ~4-6 hours light adaptation) phases of cone photopic vision. Original findings were uncovered in relation to cone photopic vision, including: *i*) late photopic vision is significantly impaired by fenretinide, but not by emixustat, *ii*) immediate photopic vision is reliant on Rpe65, *iii*) 30 mins of light is sufficient to restore early photopic vision and surmount the RPE65 inhibitor emixustat *iv*) the RPE65-independent recovery of early photopic vision occurs with low levels of 11cRAL, *v*) exogenous 9cRAL is sufficient to

restore photopic vision and *vi*) photopic vision does not rely on *des1* or RPE-expressed *rlbp1b*.

## **Results**

### **Fenretinide, but not emixustat, impairs late cone photopic vision in zebrafish larvae**

To investigate the effects of visual cycle inhibitors on cone vision, we assayed the effects of pharmacological agents, previously used in human clinical trials (32,33), on vision of 5 dpf zebrafish larvae. Previous studies demonstrated that 5 dpf zebrafish rely exclusively on cones for vision (34). Emixustat is a potent Rpe65 inhibitor, while targets of fenretinide include Des1 (the putative isomerase II) (6) and retinol-binding protein 4 (Rbp4) in blood (6,35). Larvae were raised under normal cyclic light conditions (14 h light, 10 h dark) for the duration of the experiment and treated with emixustat or fenretinide from 3 dpf. Drug was replenished at 4 dpf, and OKR analysis completed at 5 dpf between ZT 2.5 – ZT 6.5 (**Figure 1A**). Surprisingly, late photopic vision was unaffected in 5 dpf larvae treated with up to 50  $\mu$ M emixustat ( $24.3 \pm 0.9$  saccades per minute) (**Figure 1B**). In contrast, larvae treated with 10  $\mu$ M fenretinide displayed significantly fewer ( $P < 0.001$ ) saccades per minute ( $7.7 \pm 0.8$ ), in comparison to vehicle controls ( $24.2 \pm 1.5$ ) (**Figure 1B**). Whole mount images reveal that 5 dpf larvae treated with emixustat or fenretinide fail to inflate their swim bladder, but otherwise have normal gross morphology (**Figure 1C**). **Figure 1D** depicts potential targets of emixustat and fenretinide in zebrafish. Zebrafish have three rpe65 isoforms, two of which are expressed in the eye (17,36). Emixustat could inhibit RPE-expressed *rpe65a* or Müller-cell expressed *rpe65c* (**Figure 1D**). Fenretinide may inhibit Des1 isomerase activity in Müller cells, or inhibit uptake of atROL from the serum by blocking Rbp4-Transferrin (TTR) interactions. In summary, in cyclic lighting conditions a broad-spectrum visual cycle inhibitor impairs late photopic vision, whereas a selective inhibitor of the canonical visual cycle did not affect vision.

### **In dark adapted zebrafish, emixustat blocks immediate photopic vision and fenretinide exerts an additive effect**

As Rpe65 is important for chromophore regeneration under dark conditions, we tested the visual cycle inhibitors in dark-adapted zebrafish larvae wherein larvae were raised under normal

cyclic light conditions until 3 dpf, then treated with drug under constant dark conditions from 3-5 dpf. Visual behavior analysis was performed at 5 dpf between ZT 2.5 and ZT 6.5, wherein larvae were individually removed from wells and immediately subjected to OKR following a two-day dark adaptation. (**Figure 2A**). Dose-response and dose-frequency studies revealed that 50  $\mu$ M emixustat added at day 3 and replaced at day 4 caused the most significant reduction in OKR saccade number/minute ( $6.8 \pm 2.1$ ,  $p < 0.001$ ) compared to vehicle controls ( $20.5 \pm 0.9$ ) (**Figure 2B**). Under dark conditions, larvae treated with 10  $\mu$ M fenretinide displayed a ~6-fold reduction in the ability to track rotating stripes, whereas larvae treated with 50  $\mu$ M emixustat had a ~2.5-fold decrease in OKR. Interestingly, when both isomerase inhibitors were combined, immediate photopic vision was completely abolished (1.2 saccades/minute,  $p < 0.001$ ) and reduced by ~22 fold, compared to vehicle controls (26.8 saccades per minute). (**Figure 2C**). Larvae treated with emixustat and fenretinide in the dark were morphologically normal, other than they did not inflate their swim bladder (**Figure 2D**) as also observed under a normal light/dark cycle (**Figure 1C**). An uninflated swim bladder is commonly observed in visually compromised zebrafish larvae (37,38). Interestingly, neither 50  $\mu$ M emixustat nor 10  $\mu$ M fenretinide induced obvious changes in retinal histology, particularly the photoreceptor outer segment structure and RPE, compared to vehicle controls (**Figure 2E-G, 2E'-2G'**). Furthermore, double cones stained with zpr-1 appear unaffected by fenretinide or emixustat treatment (**Figure 2E''-2G''**). Thus, emixustat impaired immediate photopic vision following dark adaptation (**Figure 2A and C**) but not late photopic vision (**Figure 1B**), while fenretinide inhibited both immediate and late photopic vision.

### **Visual Cycle Inhibitors Modulate the Profile of Key Retinoids in Zebrafish**

To assess the pharmacological specificity of the phenotypes in zebrafish, we measured the content of visual retinoids in zebrafish larval heads following pharmacological visual-cycle inhibition. Animals genetically or pharmacologically devoid of functional visual cycles can present with significantly altered levels of retinoids in the eye and liver (17,21,23,39-41). In agreement with the impaired immediate photopic vision we observe in zebrafish, we observed a significant reduction in

11cRAL, 11cROL and the 11cRE, 11-*cis*-retinyl palmitate (11cRP), following emixustat and fenretinide treatment, alone or in combination ( $p < 0.001$ ) (**Figure 3B**). Rpe65 uses atREs as its substrate, therefore, elevated levels could be expected in the presence of emixustat (42). Interestingly, atROL ( $23.2 \pm 2.1$  pmole/mg,  $p < 0.01$ ) and 9cRAL levels were significantly reduced ( $10.48 \pm 0.9$  pmole/mg,  $p < 0.05$ ) in fenretinide-only treated larvae compared to the vehicle control ( $70.5 \pm 15.7$  and  $20.8 \pm 1.3$  pmole/mg, respectively). These data suggest that emixustat and fenretinide impair immediate photopic vision following dark adaption in zebrafish by reducing 11cRAL levels.

#### **9cRAL supplementation significantly improves immediate photopic vision in emixustat and fenretinide-treated larvae**

As cone function is restored in *Rpe65*<sup>-/-</sup> mice following supplementation with 9cRAL, a functional analogue of 11cRAL (43), we measured cone vision following treatment with exogenous 9cRAL. Larvae were treated with emixustat and/or fenretinide under dark-adapted conditions as described above (**Figure 2A**). In addition, larvae were treated with 9cRAL four hours following treatment with emixustat and/or fenretinide at both 3 and 4 dpf (**Figure 4A**). Immediate photopic vision in emixustat-treated larvae supplemented with 9cRAL improved significantly ( $16.1 \pm 1.3$  saccades per min,  $p < 0.001$ ), compared to emixustat-treatment alone ( $10.5 \pm 0.88$  saccades per min) (**Figure 4B**). We also observed approximately two-fold ( $6.5 \pm 1$  saccades per min,  $p < 0.01$ ) improved cone vision in fenretinide treated larvae following supplementation with 9cRAL (**Figure 4B**). 9cRAL supplementation improved vision in emixustat plus fenretinide-treated larvae approximately five-fold compared to emixustat and fenretinide-treated larvae without 9cRAL supplementation (5.3 vs 1 saccade per minute, respectively) (**Figure 4B**). No gross morphological changes were observed in larvae treated with 9cRAL in combination with fenretinide alone or fenretinide and emixustat (**Figure 4C**). Interestingly, larvae treated with emixustat and 9cRAL inflated their swim bladders (**Figure 4C**), a characteristic not observed in larvae treated with emixustat alone (**Figure 2D**). Finally, treatment of larvae with exogenous 9cRAL altered retinoid profiles at 5 dpf (**Figure 4D**). All 9-*cis* retinoids (9cRP, 9cROL and 9cRAL) and atRP significantly

increased in emixustat and 9cRAL-treated larvae, compared to emixustat alone. Presence of small amounts of 9cRAL or 13cRAL in samples not treated with exogenous chromophore is most likely a result of thermal isomerization (**Supplementary Figure 1**). Interestingly, no significant increase was observed in 11cRAL profile following 9cRAL supplementation. Thus, the reduction in immediate photopic vision in emixustat and fenretinide-treated larvae results not from toxic effects, but rather from visual cycle inhibition.

#### **Early photopic vision recovers in emixustat-treated larvae following exposure to light**

Recent studies proposed the non-canonical visual cycle to rely on a Rpe65-independent isomerization event which relies on RGR-opsin/Rdh10 and occurs during sustained exposure to visible light (44). We hypothesized that exposure of larvae to light for 30 minutes following a prolonged dark adaptation may activate the alternative pathway and modulate the impaired visual behavior in larvae treated with emixustat and fenretinide under dark-adapted conditions (**Figure 2C**). At 5 dpf, between ZT 2.5 – ZT 6.5, larvae were subjected to the OKR assay immediately after dark adaptation for two days (0 minutes) or following 30 minutes subsequent exposure to ambient light (**Figure 5A**). Pre-treatment with 50  $\mu$ M emixustat partially suppressed immediate photopic vision in dark-adapted larvae ( $11.4 \pm 1.7$  saccades per minute,  $p < 0.001$ ) compared to vehicle controls ( $17.3$  saccades per minute) (**Figure 5B**). In contrast, pre-treatment with 10  $\mu$ M fenretinide virtually abolished immediate cone photopic vision in dark-adapted larvae ( $1.7 \pm 0.6$  saccades per minute,  $p < 0.001$ ) compared to the vehicle control ( $17.3 \pm 0.8$  saccades per minute) (**Figure 5B**). To help discriminate if fenretinide acts via Rbp4 or Des1, A1120, a potent Rbp4 inhibitor was tested. Similar to fenretinide, A1120 suppressed immediate cone photopic vision ( $10.2 \pm 1.2$  saccades per minute). Subsequent exposure of untreated larvae to 30 minutes of light had minimum effect on early cone visual function versus untreated, dark-adapted larvae (**Figure 5B**).

Interestingly, early photopic vision in emixustat-treated larvae was indistinguishable from vehicle-only treated larvae after 30-minutes exposure to light (**Figure 5B**), despite lower 11cRAL levels in larvae treated with emixustat (**Figure 5C**). Pre-treatment of larvae with fenretinide virtually

abolished cone vision following 30 minutes of light exposure, similar to fenretinide-treated, dark-adapted larvae (**Figure 5B**). Surprisingly, early photopic vision in A1120-treated larvae recovered to vehicle control levels ( $21 \pm 1$  saccades per minute) indicating that the block in early photopic vision observed following fenretinide treatment is not a result of inhibiting Rbp4 (**Figure 5B**).

The relatively normal cone OKRs in emixustat-treated larvae following light exposure raised the question if this correlated with increased 11cRAL chromophore levels. Retinoid levels were profiled in emixustat-treated larval heads at 0 and 30 minutes of light exposure (**Figure 5C**). Although vision recovered following 30 minutes light exposure (**Figure 5B**), 11cRAL levels surprisingly did not change. Notably, 9cRAL levels were significantly reduced in the dark ( $11.6$  pmole/mg,  $p < 0.05$ ), compared to its respective vehicle control ( $20.8 \pm 1.3$  pmole/mg) and recovered to levels similar to the vehicle control in the light ( $14.9 \pm 1.3$  vs  $15.9 \pm 0.7$  pmole/mg). However, as 9cRAL abundance is naturally low in the zebrafish eye, it is unlikely that it mediates the recovery of vision following 30 minutes light exposure. Interestingly, atRAL levels returned to that of the vehicle control following 30 minutes indicating photoisomerization of light sensitive chromophore and activation of the phototransduction cascade.

As early photopic vision, in the presence of emixustat, recovered following exposure to ambient light for 30 minutes, there was a possibility of light-induced degradation or inactivation of emixustat. To test this possibility, we performed mass spectrometry analysis of emixustat before and after light exposure. No change in the relative abundance of the ion corresponding to emixustat ( $m/z = 263.81$ — $264.26$ ) was observed (**Figure 5D**). NMR analysis also demonstrated no chemical change to emixustat upon exposure to visible light. (**Figure 5E**).

To confirm that early photopic vision was not mediated by reactivation of RPE65, larvae were treated in the dark with emixustat from 3 dpf and emixustat was removed at 5 dpf under dim red light, 30 minutes before OKR analysis at ZT 2.5- ZT 6.5. OKR was conducted at 0- and 30-minutes post light exposure (**Figure 6A**). As before, emixustat-treated larvae presented with a significantly reduced immediate photopic vision following dark adaptation ( $13.8 \pm 1.1$  saccades

per minute,  $p = 0.0003$ ) which recovered 30 minutes post light exposure ( $23.4 \pm 1.2$ ,  $p < 0.0001$ ) but not following 30 mins of removal of emixustat alone (**Figure 6B**). Retinoid analysis again revealed a reduction in 11cRAL which did not recover following a 30-minute absence of emixustat and/or 30 mins light exposure (**Figure 6C**). A significant increase in atRP in emixustat-treated larvae in both 0- and 30-minute groups reaffirms Rpe65 inhibition (**Figure 6C**). In summary, while emixustat-treated wildtype larvae display impaired immediate photopic vision, larvae can regain vision following 30 minutes of light exposure. This light-dependent recovery of early photopic vision is not due to Rpe65 isomerase activity.

### **Des1 is not required for cone photopic vision in zebrafish at 5 dpf**

Previous studies implicated DES1 as the isomerase II in regeneration of cone visual pigments through the intraretinal visual cycle (6). To test if *des1* is an important modulator of cone photopic vision, we created *des1*<sup>-/-</sup> zebrafish using CRISPR/Cas9 by engineering a deletion in exon 2 (**Figure 7A**). No gross morphological differences were observed in *des1*<sup>-/-</sup> larvae at 5 dpf, compared to their unaffected siblings (**Figure 7C**). Late photopic vision at ZT 2.5 – ZT 6.5 is not significantly affected in *des1*<sup>-/-</sup> larvae at 5 dpf compared to unaffected *des1*<sup>+/+</sup> or *des1*<sup>+/-</sup> siblings (**Figure 7D-E**). We randomly drug treated offspring from a *des1*<sup>+/-</sup> incross with emixustat and incubated in dark (**Figure 7F**). Interestingly, neither immediate nor early photopic vision is significantly affected between the populations treated with vehicle or 50  $\mu$ M emixustat (**Figure 7G**). A genotyped sample of each group validated the normal photopic response of *des1*<sup>-/-</sup> larvae (**Figure 7H**).

### **RPE-expressed zebrafish *rlbp1b* is not required for cone photopic vision**

Co-immunoprecipitation experiments revealed that *RLBP1*-encoded CRALBP interacts with DES1 and increases the rate of 11cROL synthesis (6). Zebrafish *rlbp1* has sub-functionalized, with *rlbp1a* expressed in Muller cells and *rlbp1b* expressed in the RPE (20,21). Here, a zebrafish knockout of RPE-expressed *rlbp1b* was created using CRISPR/Cas9 which introduced a 1440 bp deletion across exon 2 and 3 (**Figure 8A**). We found that following *rlbp1b* knockout, *rlbp1b* gene expression was completely abolished ( $P < 0.0001$ ) in *rlbp1b*<sup>-/-</sup> F2 larval eyes. (**Figure**

**8C).** Gross morphology of *rlbp1b*<sup>-/-</sup> larvae is identical to their siblings at 5 dpf (**Figure 8D**). Previous studies report a reduction in cone vision and function following *Rlbp1b* knockdown (20,21) however here, no differences in immediate, early or late photopic vision were observed (**Figure 8E-F**).

### Discussion

Many daily tasks are dependent on the ability of the eye to functionally adapt to changes in light. Vision in well-lit environments is known as photopic vision and is mediated by cone photoreceptors (45). However, little is known regarding the biological processes underpinning functional photopic vision. Here, we apply chemical biology and genetics to dissect the contribution of light, retinoids, retinoid carrier proteins and retinoid isomerases to photopic vision *i)* immediately following dark adaptation, *ii)* after 30 minutes of light or *iii)* after ~4-6 hours of light. We exploited emixustat for its chemical ability to scavenge retinal and inhibit the Rpe65 isomerase, (23). Likewise, fenretinide causes depletion of vitamin A by disrupting the retinol-dependent binding of RBP4 to transthyretin, (22), and also inhibits the Des1 isomerase (46). A1120 is a more-selective RBP4 antagonist.

### **Immediate and late cone photopic vision depend on different visual cycle pathways**

The ray-finned fish lineage, including zebrafish, underwent whole-genome duplication resulting in two paralogues of many teleost genes (47). In zebrafish, Rpe65a is exclusively expressed in the RPE whereas a contentious Rpe65c isoform was localized to retinal Müller glia (36). Unexpectedly, larvae treated with emixustat and reared on a light/dark cycle presented with normal cone-based vision at late-morning/mid-day (ZT 2.5 – ZT 6.5). This agrees with previous genetic studies demonstrating OKR contrast sensitivity is unaffected in Rpe65a knockdown larvae raised under normal lighting conditions (17). Thus, under prolonged photopic conditions, zebrafish Rpe65 appears redundant for photopic vision, suggesting an alternate mechanism provides chromophore for cone visual pigments.

In cones, both RPE and intraretinal visual cycles are required to fully complete dark adaptation (48). Therefore, we hypothesized that emixustat would impair immediate photopic vision following dark adaptation. Indeed,

emixustat-treated larvae display reduced immediate photopic vision, whereas vehicle controls show full responses. We propose that emixustat impairs immediate photopic vision by inhibiting Rpe65a activity in the zebrafish RPE. However, we cannot unequivocally exclude a role for Rpe65c in zebrafish Müller glia.

### **Retinoid profiles are altered in dark-adapted zebrafish larvae treated with visual cycle isomerase inhibitors**

Retinoid analysis from dark-adapted zebrafish larvae show reduced 11-*cis*-retinoids (11cROL, 11cRAL and 11cRP) with emixustat-treatment. The large change in 11cRAL was not matched by a commensurate increase in atRP levels. Fenretinide also dose-dependently suppressed immediate photopic vision. However, although 11-*cis*-retinoid levels were lower in fenretinide- versus vehicle-treated larvae, the degree of suppressed vision was less than with emixustat. Levels of atROL, atRAL and atRP were also suppressed in fenretinide- versus vehicle-treated larvae, an effect not observed in emixustat-treated larvae. Two non-mutually exclusive mechanisms may explain these effects. The first is a vitamin A-deficiency state induced by fenretinide binding to RBP4 (49). This should globally reduce visual retinoids, as observed here. The second is fenretinide inhibition of Des1. If Des1 acts in conjunction with MFAT (50), it should reduce levels of all three 11-*cis*-retinoids, as observed.

### **Exogenous 9cRAL supplementation restores immediate photopic vision following visual cycle inhibition**

Treatment with exogenous 9cRAL recovers ERG responses in *Rpe65*<sup>-/-</sup> mice and functional vision in *Rpe65*-deficient dogs, establishing its potential as a chromophore replacement therapy (42,51,52). In agreement, immediate photopic vision significantly improved emixustat and/or fenretinide-treated larvae supplemented with 9cRAL. Thus, the impaired cone vision we observe is a specific pharmacological effect on the visual cycle and not a result of ocular toxicity. Additionally, fenretinide-treated larvae display impaired immediate photopic vision despite relatively high 11cRAL levels. The improvement of vision with 9cRAL suggests that 11cRAL stores present may be unavailable to cones in fenretinide-treated larvae. Improved vision in emixustat-treated larvae supplemented with 9cRAL was not



coupled to increased 11cRAL levels. Instead, all 9-*cis* retinoid profiles (9cRAL, 9cROL and 9cRP) were significantly increased, indicating that vision is mediated by iso-opsins.

### **Differential requirements of Rpe65 for immediate and early cone-based photopic vision**

Since emixustat did not affect late photopic vision in zebrafish, we investigated if impaired immediate photopic vision in dark-adapted animals could recover in a light-dependent, Rpe65-independent manner. Intriguingly, diminished vision in dark-adapted larvae recovers after 30 minutes of light exposure, even in the presence of emixustat. This result, together with emixustat-treated larvae displaying normal late photopic vision, suggests early- and late-phases of photopic vision are mediated by an alternative, light-driven response. The alternative explanation of a loss of emixustat activity is unlikely as: *i*) no structural alterations to emixustat occurred following 30 mins of light exposure and *ii*) an equivalent visual impairment is observed if emixustat is removed 30 minutes prior to conducting OKR measurements. Notably, electrophysiological studies in rod and cone dominant species demonstrate sustained cone responsiveness during periods of prolonged light exposure in the presence of the potent Rpe65 inhibitors, Ret-NH<sub>2</sub>, MB-001 and emixustat reaffirming a Rpe65-independent isomerase regenerates cone visual pigments (28). Pharmacological RPE65 inhibition significantly reduces 11cRAL levels in multiple models *e.g.* by Ret-NH<sub>2</sub> in zebrafish (17), by emixustat in mice (23) and by emixustat or fenretinide in *ex vivo* RPE microsomes (28). Here as expected, dark-adapted, wildtype larvae treated with emixustat display a ~10-fold reduction in 11cRAL. Interestingly, 11cRAL levels did not recover following 30 mins light exposure despite recovery of early photopic vision. A reduction in total retinoid was observed indicating that emixustat may be trapping atRAL and forming unknown or undetectable conjugates. Consistent with light overcoming emixustat-mediated visual impairment, atRAL was restored to levels similar to vehicle controls 30 minutes following light exposure, consistent with initiation of phototransduction. However, that photopic vision recovers in the light, surmounting emixustat, without an associated increase in 11cRAL levels is intriguing. Potentially, the small amount of 11cRAL available to the photopic cycle activates

cone opsins in a steady state and is sufficient to sustain a stable visual response during this 30-minute light exposure. In *Rpe65*<sup>-/-</sup> mice, a rod response remains in the absence of 11cRAL, thought to be mediated by isorhodopsin, a rod pigment formed with 9cRAL (53). Although *Rpe65*<sup>-/-</sup> mice accumulate high levels of 9cRAL under dark adaptation (53), we did not observe significantly elevated levels of 9cRAL isomers in emixustat-treated larvae following dark adaptation. The low abundance of 9-*cis* retinoids implies it is unlikely to contribute to the recovered vision post light exposure. As REs support cone function (54) and are a significant source for cone pigment regeneration (55) one possibility is that 11cRP reserves were utilized upon exposure to light. However, in line with previous studies employing RPE65 inhibitors in zebrafish (54), emixustat treatment completely blocked generation of 11cRP both in the dark and light. An alternative RPE65-independent isomerization event for cone pigment regeneration was recently described in mice (44). This cone regeneration mechanisms involves Müller glia-expressed RGR-opsin, a reversible retinol dehydrogenase RDH10, which converts atROL to 11cROL upon visible light exposure. In RPE cells, RGR opsin also affects light-dependent mobilization of atREs, suggesting it diverts substrate away from Rpe65 in RPE cells toward the Müller cell visual cycle (56).

Using genetic and pharmacological approaches, we investigate the molecular mechanisms mediating this 30-minute switch from a light-independent visual cycle, supporting immediate photopic vision, to a light-dependent visual cycle, supporting early photopic vision. When wildtype larvae were treated with the potent RBP4 antagonist A1120 alone immediate photopic vision was reduced but recovery of early photopic vision was equivalent to vehicle controls. This indicates that RBP4 is required for the non-photopic visual cycle but not for the photopic visual cycle. Furthermore, knockout of the *des1*<sup>-/-</sup> isomerase in zebrafish showed no modulation of immediate, early or late photopic vision suggesting Des1 is not essential for either the photopic or non-photopic visual cycles. Mammalian CRALBP forms a complex with DES1 (6) and is reported to regulate the cone visual cycle. Furthermore, deletion of CRALBP in mice results in reduced photopic ERG responses (57). Adenovirus-mediated delivery of Müller cell CRALBP restored cone-driven

responses. Knockout of the RPE-expressed *rlbp1b* paralogue did not affect immediate or early photopic vision in larvae treated with emixustat.

Significantly, fenretinide and emixustat hydrochloride have reached phase II and III clinical trials, respectively, for use in Geographic atrophy. Night blindness and dry eye are side-effects of fenretinide treatment (58). Our data demonstrates that fenretinide also impairs photopic vision, thus, concerns arise over potential adverse drug reactions in the clinic with fenretinide use to treat diseases such as geographic atrophy, cancer, acne, cystic fibrosis and psoriasis. Likewise, emixustat is now in Phase III clinical trial for the inherited retinal degeneration Stargardt disease with the intention of suppressing retinoid cycling (33). We have shown that the photopic visual cycle overcomes emixustat which may have significant impact on clinical outcomes.

In summary, pharmacological and genetic models of visual cycle modulation enhanced our knowledge of the fundamental mechanisms of cone photopic vision. Differential stages of vertebrate photopic vision demand differential biochemical pathways, in light and dark. Rpe65 regenerates photopigment required by cones for immediate photopic response. In contrast, during sustained light, Rpe65 appears redundant to a light-dependent regeneration of visual pigments, a process whereby diminished 11cRAL levels are sufficient for photopic vision.

## Experimental Procedures

### Zebrafish Breeding and Maintenance

Adult zebrafish were maintained in a 14-hour light, 10-hour dark cycle in a recirculating water system at 28°C. Annual facility environmental parameters are published online: [dx.doi.org/10.17504/protocols.io.3jggkjjw](https://doi.org/10.17504/protocols.io.3jggkjjw).

Wildtype (Tübingen) larvae were generated through natural spawning. Larvae were raised at 28°C in embryo medium (0.137 M NaCl, 5.4 mM KCl, 5.5 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.44 mM KH<sub>2</sub>PO<sub>4</sub>, 1.3 mM CaCl<sub>2</sub>, 1.0 mM MgSO<sub>4</sub> and 4.2 mM NaHCO<sub>3</sub>) containing methylene blue (Sigma Aldrich, UK).

### Ethics Statement

All experiments with zebrafish larvae were performed according to ethical exemptions granted by the UCD Animal Research Ethics

Committee (AREC-Kennedy) and the Health Products Regulatory Authority (Project authorization AE18982/P062).

### Generation of mutant zebrafish

Two custom crRNA guides, containing a NGG protospacer adjacent motif (PAM) were designed per gene using Benchling ([www.benchling.com](http://www.benchling.com)), CRISPR guide software. All guide sequences and PAMs used are listed in *Supplementary Figure 2*. The Alt-R system (Integrated DNA Technologies) was used to knockout *rlbp1a*, *rlbp1b* and *des1* in zebrafish. Briefly, 36 ng/ul crRNA and 67 ng/ul tracrRNA was complexed in nuclease-free duplex buffer by heating to 95°C for 10 minutes. Sample was cooled to room temperature and a final working concentration of 0.5 ug/ul *S. pyogenes* Cas9 nuclease (Integrated DNA technologies). The mixture was heated to 37°C for 15 minutes before microinjection into wildtype (Tü) zebrafish embryos at one-cell stage. Injected fish were raised until adulthood and outcrossed to screen for germline transmission of the null target gene. Isogenic F<sub>1</sub> heterozygous adult fish were incrossed to generate homozygous F<sub>2</sub> mutants.

### Genomic DNA extraction and polymerase chain reaction

DNA was extracted from injected F0 or germline F2 *rlbp1b* larvae by boiling the sample at 95°C in NaOH (50 mM) (Sigma Aldrich, UK) before neutralizing with 1/10th Trizma Base (Sigma Aldrich, UK). The region of interest was amplified by polymerase chain reaction (PCR) using the following primers which span the *rlbp1b* or *des1* mutation site: *rlbp1b* 5'-CCACAGAGTGGAACATTTCTGA-3' and 5'-ACCTTTCAGCATTACAAATCCT-3'; *des1* 5'-TTCTCCATGACCTTTCAGCCCC-3' and 5'-TGCTCTCACCATTGGTAGTCG-3'. Fragment sizes were run on a 2% agarose gel and compared against a 100 bp DNA ladder.

### Total RNA extraction, cDNA synthesis and qPCR

At 5 dpf, ~20-50 eyes (10-25 larvae) were enucleated and stored in RNAlater (Qiagen, Germany) individually at 4°C. Larval trunks were genotyped as previously described, eyes were pooled and total RNA was extracted from eyes using mirVana™ miRNA Isolation Kit (ThermoFisher Scientific) as per the manufacturer's instructions. 10µl 3M NaOAc and 220µl 100% ethanol was added to the eluted

RNA, vortexed and left overnight at -20°C. Samples were centrifuged at 14000 rpm for 60 minutes at 4°C, supernatant removed before the pellet was resuspended in 80% ethanol and centrifugation for a further 60 minutes at 14000 rpm. Supernatant was discarded and the pellet was resuspended in 20 µl ultrapure water. Total RNA concentration was quantified at 260 nm (Spectrophotometer ND-1000) and samples stored at -80°C until further use. cDNA was synthesized using PrimeScript™ RT reagent Kit (Perfect Real Time) (TAKARA, Japan). QRT-PCR reactions were made up on ice with 0.2 µl forward and reverse primer: *rlbp1b* 5'-TGAGCTTGCTAAAGGTGTTTCAGG-3' and 5'-TCAGGATAATCCCGTCTGAAGC-3'; *des1* 5'-GGACGTGGACATCCCCACTGA -3' and 5'-AGCTGGATGGCCACGTTCAG -3' 5 µl Sybr Green Master Mix (Thermo Fisher Scientific, Massachusetts, USA), 3.6 µl RNase-free water and 1 µl cDNA template (12.5 ng). QRT-PCR cycles were carried out using the following parameters: 50°C for 2 min, 95°C for 10 min, 95°C for 15 s with 40 repeats and 60°C for 1 min on a Quant Studio 7 Flex Real-Time PCR System.

#### Drug Preparation and Treatment

Wildtype (Tübingen), *rlbp1a*<sup>-/-</sup>, *rlbp1b*<sup>-/-</sup> and *des1*<sup>-/-</sup> larvae were raised from embryos under standard 14-hour light, 10- hour dark conditions. At 3 dpf, five larvae per well were placed in 48 well cell culture plates (Greiner Bio-one, Austria). Emixustat hydrochloride (Medchem, New Jersey, USA), fenretinide (Cayman Chemical, Michigan, USA) or A1120 were prepared in embryo medium and 400 µl drug solution at 1-50 µM in 0.01%- 0.5% DMSO added wells. For emixustat and fenretinide combinations, 1-10 µM fenretinide was added 15 mins prior to the addition of 50 µM emixustat. Larvae were initially treated at 74-76 hpf and the drug was replaced at 98-100 hpf. 10 µM 9-*cis*-retinal (Sigma Aldrich, UK) was added to embryo medium four hours following primary drug treatment (*i.e.* 78-80 hpf and 102-104 hpf). 9-*cis*-retinal was handled under dim red light.

#### Visual Behavior Analysis

To measure optokinetic response (OKR), single larvae were immobilized in pre-warmed 9% methylcellulose (Sigma Aldrich, UK) and placed in the center of a rotating drum containing vertical black and white stripes. The drum was rotated at 18 rpm for 30 seconds in a clockwise and 30 seconds in a counterclockwise direction. Saccadic

eye movements per minute were recorded manually (59). All OKR measurements were performed in larvae at 5 dpf between 10 am (ZT 2.5) and 2 pm (ZT 6.5).

#### Histological Analysis

Larvae were fixed in glass vials containing 2.5% glutaraldehyde, 2% paraformaldehyde and 0.1% Sorenson's phosphate buffer (pH 7.3) and were placed at 4°C. Samples were washed before post-fixation in 1% osmium tetroxide (Sigma Aldrich, UK) and dehydrated using an ethanol gradient (30%, 50%, 70%, 90%, 100%). Samples were transferred to propylene oxide (Sigma Aldrich, UK), embedded in agar epoxy resin overnight and 0.5 µm sections obtained using an ultramicrotome (Leica EM UC6). Sections were placed on glass slides and stained with Toluidine blue (Sigma Aldrich, UK) for 30-60 seconds. Samples were imaged using a Leica DMLB bright field illumination microscope with a Leica DFC 480 camera attachment.

For transmission electron microscopy (TEM), 0.08 µm sections were cut using a diamond knife and a Leica EM UC6 ultramicrotome. Sections were transferred to a support grid, stained with uranyl acetate and lead citrate, and imaged on a FEI-Tecnai 12 BioTwin transmission electron microscope (FEI Electron Optics). Photoreceptor outer segment area was measured manually using ImageJ software.

Drug treated larvae were fixed at 5 dpf with 4% paraformaldehyde (PFA; Sigma Aldrich, UK). Larvae were cryoprotected in a sucrose series before embedding in optimal cutting temperature (OCT) medium (VWR, Pennsylvania, United States). 14 µm sections were cut and thaw-mounted onto charged superfrost plus slides (Fisher Scientific, Pittsburgh, USA). Sections were rehydrated using 1X phosphate buffered saline supplemented with tween 20 (Sigma Aldrich, UK) (0.01% PBST) before blocking in 10% goat serum (Sigma Aldrich, UK) for 1 hour. Monoclonal zpr-1 was used at 1:200 (Zebrafish International Resource Center, Eugene, USA). Slides were washed before incubating with the secondary antibody (Alexa Fluor 488 anti-mouse IgG). Primary and secondary antibodies were diluted in blocking solution. Slides were mounted using Vectashield; (Vector Laboratories, California, USA) before imaging using a Zeiss LSM510 confocal microscope (Carl Zeiss AG, Germany) with a 63X objective.

### Retinoid Quantification

For retinoid analysis, 105 larvae per treatment condition at 5 dpf were euthanized in ice water before their heads were collected under dim red light. For normal-phase HPLC analysis of retinoids, all retinoid extractions were carried out under dim red light in a dark room. Zebrafish heads were stored at  $-80^{\circ}\text{C}$ , thawed on ice, and homogenized in 500  $\mu\text{L}$  of phosphate buffered saline (PBS) using a glass tissue grinder (Kontes). 50  $\mu\text{L}$  samples were processed for protein concentration determination using the Micro BCA Protein Assay Kit (Pierce, US). To the remaining 450  $\mu\text{L}$  of homogenate 25  $\mu\text{L}$  of 5% SDS ( $\sim 0.2\%$  SDS final concentration) and 50  $\mu\text{L}$  of brine were added and samples briefly mixed. Hydroxylamine hydrochloride (500  $\mu\text{L}$  of 1.0 M solution in pH 7.0 PBS) was added to generate retinal oximes and samples were vortexed and incubated at room temperature for 15 minutes. 2 ml of cold methanol was added to quench and dilute the aqueous phase. The samples were twice extracted by addition of 2 ml aliquots of hexane, brief vortexing, and centrifugation at 3000 x g for five minutes to separate the phases. Pooled hexane extracts were added to 13 x 100 mm borosilicate test tubes and evaporated to dryness under a stream of nitrogen. Dried samples were dissolved in 100  $\mu\text{L}$  hexane and analyzed by normal-phase HPLC using an Agilent 1100 series chromatograph equipped with a Supelcosil LC-Si column ( $4.6 \times 250$  mm, 5  $\mu\text{m}$ ) using a 0.2–10% dioxane gradient in hexane at a flow rate of two mL per minute. The eluted mobile phase was analyzed using a photodiode-array detector. Spectra (210–450 nm) were acquired for all eluted peaks. The identity of eluted peaks was established by comparison to spectra and elution times of known authentic retinoid standards. Retinoid amounts were quantitated by comparing their respective peak areas to calibration curves established with retinoid standards.

### Photostability of Emixustat Hydrochloride

Liquid chromatography used a Dionex Ultimate 3000 RSLC micro-LC system (Thermo Fisher Scientific, US). Separation was performed using a Thermo Acclaim RSLC 120 2.2  $\mu\text{m}$ , 120 A, 1.0 x 100 mm LC column (Thermo Fisher Scientific, US). LC parameters were: elution mode: gradient (from 90% A to 90% B in 15 min), flow rate: 45  $\mu\text{L}/\text{min}$ , column temperature:  $3^{\circ}\text{C}$ , detector wavelength: 230 nm and injection volume: 2  $\mu\text{L}$ .

Mobile phases were: A:  $\text{H}_2\text{O}$  + 0.1% formic acid (FA) (v/v) B: Acetonitrile + 0.1% FA (v/v) Mass Spectrometry analysis was performed using a LTQ-XL-Orbital-XL (Thermo Fisher Scientific, US) equipped with HESI II ESI ion source. Source parameters were: Ion spray voltage (V): 4.6, capillary Temp ( $^{\circ}\text{C}$ ): 280, sheath gas flow gas flow (Arb): 20 and auxiliary gas flow (Arb): 8. Resolution was set to 30,000 in FT mode and mass range set to 150–500 Da in positive ion mode. Peak area of emixustat protonated mass was calculated and recorded using Xcalibur software (version 2.21). The protonated mass ions were identified using an error tolerance of 5 ppm for their corresponding theoretical monoisotopic mass.

For Nuclear Magnetic Resonance (NMR) analysis, emixustat (7 mg) was dissolved in DMSO- $d_6$  (600  $\mu\text{L}$ ) under dim red light and covered with aluminum foil until  $^1\text{H}$  NMR and HSQC experiments were performed. The NMR tube was exposed to visible light for 30 minutes, followed by a repeat NMR analysis.  $^1\text{H}$  NMR spectra were measured in the solvent stated at 400 MHz. Chemical shifts ( $\delta$ ) are quoted in parts per million (ppm) referenced to residual solvent peak (e.g., DMSO- $d_6$ :  $^1\text{H}$  – 2.50 ppm and 3.33 ppm).

### Statistical Analysis

Statistical analysis was performed using GraphPad Prism<sup>TM</sup> software (GraphPad, San Diego, CA). A two-tailed student's unpaired t-test was applied comparing two experimental groups. For analysis involving more than two independent groups, a one-way ANOVA and Dunnett's multiple comparison test or Bonferroni post hoc test was performed. Experiments comprised of two variables were tested for significance using a two-way ANOVA and a Bonferroni post hoc test. All data are presented as mean  $\pm$  standard error of the mean (SEM). Statistical significance was assigned to p-values of  $p < 0.05 = *$ ,  $p < 0.01 = **$  or  $p < 0.001 = ***$ .

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### Conflicts of interest

The authors declare that they have no conflicts of interest with the contents of this article

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#### Abbreviations

AMD - Age-related macular degeneration  
atROL – all-*trans*-retinol  
A2E - *N*-retinylidene-*N*-retinylethanolamine  
atRE – all-*trans*-retinyl esters  
atRP - all-*trans*-retinyl palmitate  
DES1 - Dihydroceramide desaturase-1  
LRAT - Lecithin retinol acyltransferase  
OKR – Optokinetic Response  
RPE – Retinal Pigment Epithelium  
RBP4 - Retinol-binding protein 4  
ZT – Zeitgeber  
9cRAL – 9-*cis*-retinal  
11cRAL - 11-*cis*-retinal  
11cROL – 11-*cis*-retinol  
11cRE - 11-*cis*-retinyl ester  
11cRP – 11-*cis*-retinyl palmitate  
13cRAL – 13-*cis*-retinal